INTERNAL BREAKDOWN OF RED CLOVER (*TRIFOLIUM PRATENSE L.*) IN RELATION TO ENVIRONMENTAL, CULTURAL, AND GENETIC FACTORS

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INTERNAL BREAKDOWN OF RED CLOVER (*TRIFOLIUM PRATENSE* L.) IN RELATION TO ENVIRONMENTAL, CULTURAL, AND GENETIC FACTORS[1/]

by

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INTRODUCTION

Red clover is an excellent hay and pasture legume. However, its use has declined because of depletion of stands during and after the second year of growth. Root and crown diseases, often in combination with root insect attacks, are frequently mentioned as primary factors limiting the persistence of stands. Graham and Newton (1959)[4/] reported internal necrosis of crown pith tissue which they called internal breakdown (IB). This disorder apparently had received little attention before 1959. The necrosis first occurs when plants are 3 months or more old. It appears as a small, dark translucent area in the upper taproot near the zone of transition from root to stem tissue (fig. 1). As plants mature, the incidence of IB increases, and affected areas enlarge. Later, they become corky and dry. External symptoms of IB are not evident. Graham, Rhykerd, and Newton (1960) reported that IB was found from Maine to Virginia in every field sampled. In greenhouse studies, they found that the incidence of IB in plants increased from 23 percent at the end of 12 weeks to 72 percent at the end of 41 weeks. In a field study, Newton and Graham (1960) observed the incidence of IB during two growing seasons. They concluded that IB was a major factor in the lack of persistence of red clover.

These initial experiments emphasized the need to investigate the role of various environmental, cultural, and genetic factors in the etiology of IB. This report summarizes results of a series of experiments conducted between

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3/ Grateful appreciation is extended to R. R. Hill, Jr., research agronomist at the U.S. Regional Pasture Research Laboratory, for assistance in statistical analysis of the data.

4/ Underscored years in parentheses refer to Literature Cited on page 23.

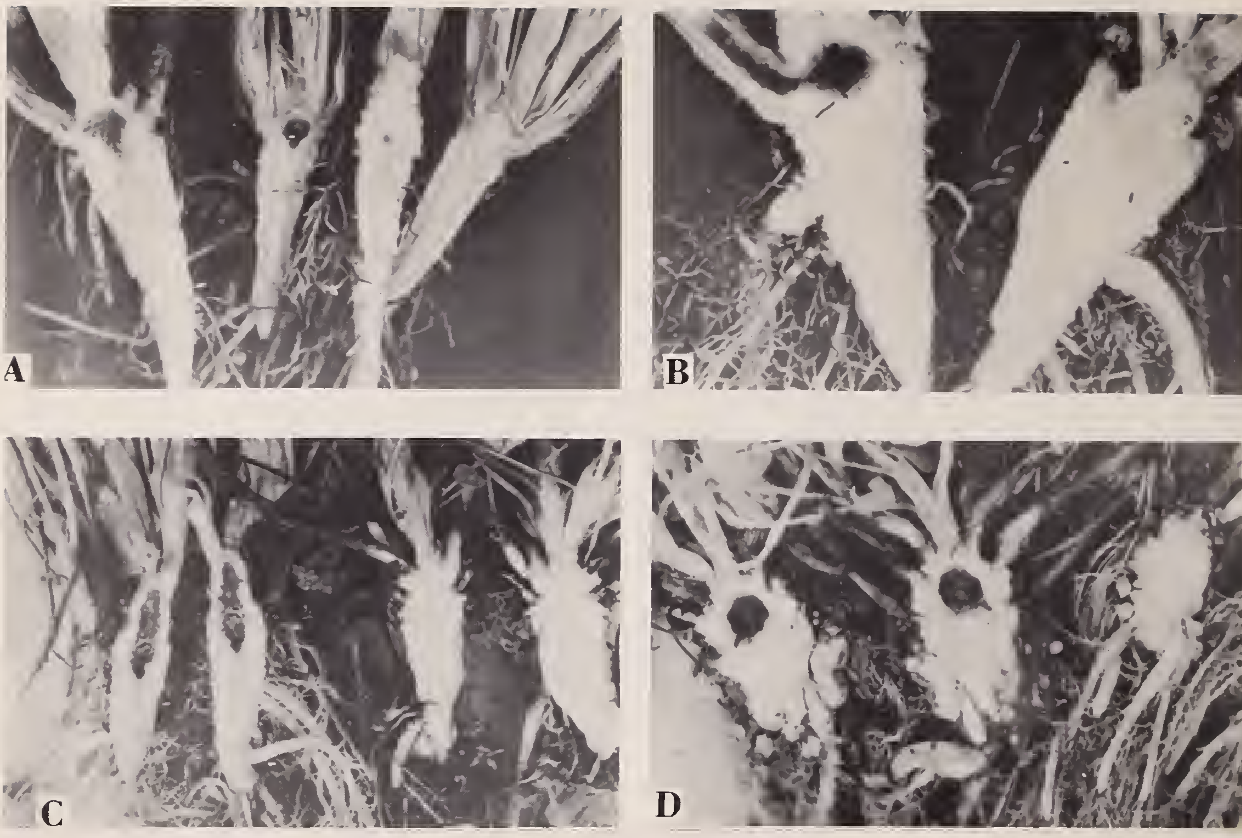

Figure 1.--Internal breakdown in red clover crowns: (A) Gradations of IB severity: Left, severe IB; right, no IB. (B) Left, IB in crown and stem; right, no IB. (C) Left, severe elongated IB area in narrow crown; right, no IB. (D) Left, severe IB in wide crown; right, no IB. Plants were grown in the greenhouse, and ranged in age from 3 to 7 months. Specimen B is slightly magnified.

1960 and 1967 to determine the cause of IB in red clover and factors affecting its development. In some instances where results were negative, only a summary statement is presented.

MATERIALS AND METHODS

Medium red clover (Pennscott variety) was used in all experiments unless otherwise indicated. The greenhouse trials were conducted at University Park, Pa., with plants grown from seed under natural day length in 4- or 6-inch pots of steamed soil fertilized with P_2O_5 and K_2O (composition: three parts Hagerstown silt loam top soil, one part sand, and one part peat moss). Plants were clipped approximately 5 cm above soil level at or near the full-bloom stage. Red clover crowns were examined for the presence of IB by washing the root systems to remove soil and by cutting lengthwise the taproots in half. Crown diameters were measured at the widest part of the taproot, just below the crown. The length and width of the IB area were recorded and used as a measure of IB severity. Exceptions to the general conditions and procedures just described will be indicated. For most experiments, an analysis of variance was used to determine the statistical significance of differences in incidence of IB.

RESULTS

Role of Micro-organisms

Isolations.--Preliminary investigations failed to implicate a pathogen as the cause of IB (Graham and others, 1960), but the possibility of pathogen involvement was not entirely eliminated. Additional isolations were made from 6-month-old plants grown in steamed potting soil and in untreated field soil. Sections of crown tissue were surface-sterilized, then broken apart, and pieces of internal tissue transferred aseptically to petri plates of "V-8 juice" agar[5]. Some pieces were from IB and contiguous tissue, while others were from non-IB plants.

Micro-organisms were not recovered from a majority of the tissue pieces (table 1). Bacteria accounted for 74 percent of the total isolations, and 93 percent of the bacterial isolations were from IB tissue.

The fungi isolated were *Chaetomium* sp. on three tissue pieces from plants in steamed soil, and *Penicillium* sp. on one piece from each of the two soils. In some instances, bacteria and fungi were isolated from the same tissue piece.

5/ Trade names are used in this publication solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over other products not mentioned.

In subsequent isolations from field plants, micro-organisms most commonly associated with IB and contiguous tissue were bacteria, Phoma sp., and Fusarium spp., in that order of frequency.

Table 1.--Isolation of micro-organisms from red clover tissue pieces, with and without IB, from plants grown in two soil sources

Soil source	Total tissue pieces plated	Pieces yielding-- No micro-organisms	Fungi	Bacteria
Untreated field soil:	Number	Number	Number	Number
IB tissue--------	30	22	0	8
Non-IB tissue----	11	10	1	1
Steamed soil:				
IB tissue--------	20	13	4	4
Non-IB tissue----	10	10	0	0

Pathogenicity of isolated micro-organisms.--In two greenhouse experiments, 11-week-old red clover plants were inoculated with Fusarium spp., Phoma sp., and Chaetomium sp. grown on corn meal-sand medium by mixing the inoculum with steamed soil. Plants were also inoculated by dipping the crown and roots in conidial and mycelial suspensions of these fungi. In a third experiment, bacteria isolated from crown tissue with and without IB were either injected into crowns of 7-week-old red clover plants with a hypodermic needle, or a bacterial suspension was poured over the crowns. Noninoculated control plants were included in each experiment. A total of 312 plants for the three experiments were examined for IB and root rot 4 to 5 months after inoculation. Results indicated no significant differences in incidence of IB between plants inoculated with fungi and bacteria and noninoculated plants. A few plants inoculated with Fusarium spp. died.

Development of IB in a controlled environment.--IB-susceptible plants were grown from seed in two flexible-film isolators under controlled environmental conditions for 197 days. As reported in a later section, IB-susceptible and IB-resistant plants were obtained by selection. The rooting medium was a 1:1 mixture of quartz sand and perlite. The isolators were located in an air-conditioned room with a temperature range of 16° to 24° C and a 12-hr.

photoperiod of 450 ft.-c. of cool white fluorescent light. Aseptic conditions were established at the start of the experiment. Petri plates of potato-dextrose and nutrient agar were exposed periodically inside the isolators to test for fungal or bacterial contamination or both.

In isolator No. 1, a species of Cladosporium was detected after 21 days, and an unidentified white bacterium was isolated from the drainage water from one pot after 197 days. The same fungus was isolated every time, and bacteria were never detected in plates exposed to the air. Orange-red bacteria were detected in isolator No. 2 after 71 days, and white bacteria, after 105 days. Fungi, however, were never detected during the trial.

Plants were removed from both isolators and examined for IB 197 days after seeding. IB had developed in plants from both isolators (table 2). The results demonstrate that a fungus is not the causal agent, since IB developed in the absence of a fungus in isolator No. 2. IB did occur in the presence of bacteria in both isolators. Therefore, the role of bacteria in IB development remained questionable. However, in another experiment where IB-susceptible red clover was grown in an isolator for 96 days before bacterial contamination was evident, one plant had a small IB spot in the crown. This suggests that IB can develop in the absence of bacteria. It was concluded from these trials that IB can develop in red clover crowns whether fungi or bacteria are present or not. Results support the earlier conclusion (Graham and others, 1960) that IB is a physiogenic disease.

Table 2.--Crown size, incidence, and severity of internal breakdown in IB-susceptible red clover plants after 197 days of growth under controlled environmental conditions

Item	Isolator No. 1	Isolator No. 2
Plants------------------number	12	11
Mean crown diameter--------mm.	9.4	9.3
Plants with IB----------number	3	2
Mean IB size--------------mm^2	6.7	1.1

Influence of Macronutrients, Micronutrients, Soil Fertility, and Organic Matter

Some diseases caused by minor element imbalance are characterized by internal symptoms resembling IB in red clover. For example, boron deficiency causes an internal breakdown of crucifers (Walker, McLean, and Jolivette, 1941), and excessive manganese and boron deficiency are associated with internal bark necrosis of Red Delicious apple trees (Berg and Clulo, 1946; Zeiders and Fink,

1959). Similarities, such as these, prompted further studies on the relationship between minor element imbalance and the development of IB in red clover. Graham and others (1960) reported that micronutrients applied to potted Hagerstown silt loam soil tended to reduce incidence of IB in red clover crowns. In two subsequent greenhouse experiments, red clover was grown in nutrient solutions containing all known essential elements and in solutions varying widely in concentrations of Cu, Zn, Mn, B, Mo, and Fe to evaluate the consistency of any micronutrient effect. In a third experiment, these six elements were compared with Cu and Fe applied singly to fertile greenhouse soil. In all three experiments, IB developed after every treatment. Differences in incidence of IB were not statistically significant.

In further replicated studies, red clover was grown in the greenhouse on two soils, with two levels of organic matter, two levels of nitrogen fertilizer, and two forms of nitrogen. This was a factorial experiment in a split-plot design with soils as the main plots and organic matter as the sub-plots. Sub-sub-plots were rates and sources of nitrogen fertilizer. The soils were an eroded Morrison loam, very low in fertility, and fertile greenhouse potting mixture. Organic matter levels were: 50 percent cow manure by volume and no manure. The two sources of nitrogen were calcium nitrate and ammonium sulfate. Rates of nitrogen were none and 300 pp2m of soil. Incidence of IB was not significantly affected by soil fertility, manure, or nitrogen, whether applied as calcium nitrate or ammonium sulfate. Plants with IB had slightly larger crown diameters than plants without IB from the same treatments.

Mineral Element Composition of Plant Tissues From Red Clover, With and Without IB

Leaflets and stems from plants, with and without IB, grown in nutrient solutions containing normal and supplemental levels of micronutrients were analyzed spectrochemically for P, K, Ca, Mg, Mn, Fe, Cu, B, Al, Na, and Mo. Differences in mineral nutrient content between plants with and without IB generally were small, with little evidence of either a cause or effect relationship between mineral nutrition and development of IB.

Strains of Pennscott red clover selected for resistance and susceptibility to IB were collected from field plots on Hagerstown silt loam soil. Plants were thoroughly washed to remove soil, and separated into leaflets, petioles, and crowns. The IB susceptible strain was separated further into plants with and without IB.

Results of the spectrochemical analyses of the different plant parts for 11 elements are shown in table 3. Differences in mineral composition between susceptible and resistant plants suggest no causal mineral nutrient mechanism for IB in red clover crowns. Likewise, susceptible plants with IB showed no differences in mineral content which appear associated with development of IB.

Table 3.--Elemental composition of red clover leaflets, petioles, and crowns from susceptible plants with IB (IB), without IB (No IB), and from resistant plants without IB.

Item	Mineral element										
	P	K	Ca	Mg	Mn	Fe	Cu	B	Al	Na	Zn
	--------Percent--------				----------------Mg./g.----------------						
Leaflets											
Susceptible (IB)-----	0.29	1.70	1.82	0.32	165	209	17.0	26	247	0.9	51
Susceptible (No IB)--	.26	1.70	2.06	.32	158	227	17.0	26	289	.5	49
Resistant (No IB)----	.28	1.59	2.12	.32	177	219	17.5	26	246	.9	51
Petioles											
Susceptible (IB)-----	.20	1.73	1.82	.38	48	113	18.0	31	97	1.7	31
Susceptible (No IB)--	.19	1.66	1.64	.37	52	139	17.0	27	172	1.2	28
Resistant (No IB)----	.18	1.52	1.76	.39	52	109	15.1	28	109	1.4	26
Crowns											
Susceptible (IB)-----	.28	.89	.39	.31	32	132	12.3	19	81	.6	21
Susceptible (No IB)--	.26	.89	.37	.29	25	106	8.6	20	57	1.0	20
Resistant (No IB)----	.24	.92	.33	.27	27	90	11.3	17	67	1.1	22

Effect of Growth Substances and Other Compounds

Pennscott red clover plants were treated with various growth substances, alone and in combinations, for a 7-month period in the greenhouse. Solutions or suspensions of these substances in the concentrations listed in table 4 were poured over the red clover crowns in 25 ml. of water per plant at approximately 2- to 3-week intervals. Treatments were started 3 days after planting when seedlings emerged. The percentage of plants with IB, and the mean crown diameters of plants for each treatment, are shown in table 4. Only plants treated with gibberellic acid had significantly less IB ($P<0.05$) than did control plants.

In an attempt to overcome the small crown size observed in the above experiment, gibberellic acid was applied to 7-week-old plants at 3-week intervals (a) 6 times and (b) 3 times, beginning 9 weeks later. Indole-3-acetic acid at two concentrations and maleic acid hydrazide, a growth inhibitor, were applied at concentrations shown in table 5.

Neither gibberellic acid nor indole-3-acetic acid treatments had any significant effect on incidence of IB (table 5). Maleic acid hydrazide appeared to cause a slight reduction in IB. Treatments had little effect on mean crown diameter. Maleic acid hydrazide caused severe shortening of petioles and crinkled, mosaiclike leaves. Consequently, this greatly reduced top growth.

Temperature and Clipping Frequency

Pennscott red clover plants were grown in greenhouse sections at temperatures of 17° and 27° C. from September 29, 1965, to May 8, 1966, in 6-inch pots of steamed soil, two plants per pot. One-half of the plants at each temperature were clipped five times at 1-month intervals, and one-half were clipped twice at 3.5-month intervals. There were four treatment variables with 160 plants in each, making a total of 640 plants. The last top growth harvest was made on May 8, 1966, after 221 days of growth, when all plants were measured and examined for IB.

Low clipping frequency favored plant growth at both temperatures as indicated by larger crowns (table 6). Incidence and severity (size) of IB were significantly greater in larger crowns. Temperature had a significant effect on crown diameter and IB size, but the effect on IB incidence was nonsignificant. Clipping frequency affected the incidence and severity of IB to a greater extent than did temperature. These results indicated that incidence of IB is positively correlated to crown size.

Effect of Day Length and Flowering Type

The eight varieties or strains of red clover listed in table 7 were seeded in steamed soil, two plants to a pot in the greenhouse, on October 15, 1965. After 8 weeks of growth, 52 plants of each variety were placed under

Table 4.--Crown diameter and percentage IB of 7-month-old red clover plants treated with various chemicals

Substance	Concentration	Mean crown diameter	Incidence of IB[1/]
	Mg./liter	mm.	Percent
Gibberellic acid----	50	6.2	10
Mixture[2/]-----------	--	6.7	25
Iodoacetic acid-----	372	6.5	28
None (control)------	--	7.9	39
Pennicillin--------- and Polymyxin-----------	50 50	7.4	40
Benzimidazole------- and Thymine-----------	40 2,000	7.6	40
Indole-3-acetic acid	0.5	7.3	43
DL Serine-----------	2,000	7.5	45
Benzimidazole-------	40	8.0	47
Mean	--	7.2	36
L.S.D. at 0.05 - percent level.		--	25
L.S.D. at 0.01 - percent level.		--	N.S.

N.S. = Nonsignificant.
1/ Values are for 16-30 plants. Mean = 24. Total plants = 215.
2/ Mixture of all chemicals in concentrations listed.

Table 5.--Crown diameter and percentage IB of 6-month-old red clover plants treated with plant growth regulators

Substance	Concentration	Amount applied per plant	Times applied 1/	Mean crown diameter	Incidence of IB 2/ 3/
	Mg./liter	ml.	Number	mm.	Percent
Maleic acid hydrazide---------	1,000	25	4	10.1	36
Indole-3-acetic acid----------	1.0	50	6	9.4	41
None (control)----------------	--	--	6	9.9	47
Indole-3-acetic acid----------	.4	50	6	9.6	41
Gibberellic acid--------------	50	50	6	9.4	43
Gibberellic acid--------------	50	50	3	9.6	40
Mean	--	--	--	9.7	41

1/ At 3-week intervals.

2/ Values are for 26-29 plants. Mean = 28. Total plants = 340

3/ Differences not statistically significant, $P < 0.05$.

Table 6.--Effect of temperature and clipping frequency on crown size and incidence and severity of IB in red clover after 7 months of growth (data based on 160 plants in each treatment)

Temperature (° C.)	Clipping frequency: Times clipped	Clipping frequency: Interval	Mean crown diameter	Incidence of IB	Mean IB size[1]
	Number	Months	mm.	Percent	$mm.^2$
17	5	1	7.3	17	4.0
	2	3.5	11.3	42	12.6
27	5	1	6.8	12	3.7
	2	3.5	10.7	35	9.1

Statistical Significance of Treatment Effects[2]

	Temperature	Clipping frequency
Mean crown diameter-----	*	**
Incidence of IB---------	NS	*
Mean IB size------------	**	**

1/ IB plants only.

2/ Significant at: * $P<0.05$, ** $P<0.01$. N.S. = Nonsignificant.

Table 7.--Effect of day length on crown size, incidence of IB and growth habit of early, medium early and late flowering red clover varieties and strains. Values are based on 26 plants of each variety

Flowering group	Variety or strain	Country of origin	Age of plants	Mean crown diameter SD[1/]	Mean crown diameter LD[2/]	Incidence of IB SD	Incidence of IB LD	Mean stems per plant SD	Mean stems per plant LD
			Months	Mm.	Mm.	Percent	Percent	Number	Number[3/]
Early	Berry's	United Kingdom	5	7.2	5.3	4	4	--	--
			7	8.7	5.5	0	8	8	6
Do---	Cotswold broad red	------Do------	5	7.5	5.3	4	12	--	--
			7	9.0	6.0	4	0	9	7
Medium early	English single cut (Cotswold)	------Do------	5	7.5	5.2	4	0	--	--
			7	9.0	6.5	0	12	10	8
Do----------	Aberystwyth S.151	------Do------	5	6.9	5.0	4	8	--	--
			7	9.5	5.5	12	4	10	6
Do----------	Quinekeli	Chile	5	7.1	5.2	12	4	--	--
			7	9.0	6.0	4	8	8	6
Do----------	Pennscott	USA (Pennsylvania)	5	8.2	6.5	12	15	--	--
			7	9.5	7.0	15	31**	8	5
Late	Montgomery	United Kingdom	5	7.1	5.5	4	12	--	--
			7	9.5	6.0	10	4	12	7
Do--	Aberystwyth S.123	------Do------	5	6.7	5.0	0	0	--	--
			7	8.5	6.0	8	12	11	6

1/ Short day (SD): 10-13 hr. of natural daylight.

2/ Long day (LD) : Photoperiod extended to 16 hr. with incandescent lamps.

3/ Stems not counted.

**Significantly greater than other varieties, $P < 0.01$.

each of 2 different day lengths: (1) Short day (SD), consisting of 10 to 13 hours of natural daylight, and (2) long day (LD), where the day length was extended to 16 hours with incandescent lamps. One plant was removed from each pot 5 months after seeding and examined for IB. The remaining plants were examined 7 months after seeding.

Plants grown under long days had smaller crowns and fewer stems (table 7). Varietal differences in percentage IB between LD-plants and SD-plants were not noteworthy. Significantly, after 7 months of growth, Pennscott had more IB than the other varieties. The Chilean variety, Quinekeli, which is comparable to Pennscott in growth characteristics, was less prone to IB under the conditions of the experiment. Differences in percentage of IB among United Kingdom varieties were small, but IB tended to develop sooner in medium-early and late-flowering varieties.

Selection for Resistance and Susceptibility to IB

Results of five cycles of selection within the Pennscott variety are presented in table 8. In each cycle, plants were classified as resistant if

Table 8.--Results of five cycles of selection and crossing on incidence of IB in Pennscott red clover

Selection cycle	Crosses: IB X IB: Plants examined	Crosses: IB X IB: Mean IB	Crosses: Non-IB X Non-IB: Plants examined	Crosses: Non-IB X Non-IB: Mean IB	Unselected Pennscott: Plants examined	Unselected Pennscott: Mean IB
	Number	Percent	Number	Percent	Number	Percent
1	34	40	19	16	--	65
2	189	99	1,054	70	55	65
3	--	--	534	22	74	54
4	20	100	182	38	64	72
5	7	86	460	15	65	71
5 (Polycross)	77	80	372	9	153	42
Total or mean	327	88	2,621	39	411	56

they had no IB after 6 to 8 months, and as susceptible if IB developed after 3 months of growth in the greenhouse. Crosses were made by hand between pairs of plants in each category, except the polycross plants of cycle 5, which resulted from honeybee pollination in greenhouse compartments. As controls, unselected Pennscott plants were grown in each cycle. Results indicate that both resistance and susceptibility to IB in red clover may be obtained through breeding techniques.

Note: To determine the presence of IB at a given age, it is necessary to cut through the middle of the crown and taproot, thereby severely injuring the plant. A satisfactory technique without injuring the plant has not yet been found.

IB in Rooted Stems

Four to six stems (including some crown tissue) were separated from red clover plants with and without IB, and rooted in steamed potting soil. Growth was satisfactory, although not all plants developed typical crowns. Incidence of IB in the clonal plants after 6 to 7 months of growth in the greenhouse is shown in table 9. The data support the conclusion that incidence of IB in red clover is a genetic trait.

Table 9.--Incidence of IB in rooted red clover stems after 6 to 7 months' growth in the greenhouse

Source Plants	Stems examined	Incidence of IB
	Number	Percent
With IB	80	57**
Without IB	124	21

** Significantly different, $P < 0.01$.

IB in Red Clover Varieties

IB was not restricted to the variety Pennscott. Dollard, Midland, and Lakeland red clover varieties were also tested in the greenhouse, and after 4 months of growth, incidence of IB was similar in all varieties.

IB in Alsike Clover, *Trifolium hybridum*

Two varieties of alsike clover were grown for 34 weeks in potting soil in the greenhouse. Crowns were examined for IB at intervals as shown in table 10. In all examinations, P.I. 184687[6/], introduced from Germany, was significantly more prone to IB than common alsike.

Effect of Irradiation

Previous results suggested that IB was associated with aging of plant tissue. It has also been reported (Curtis, 1963) that radiation damage in

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6/ P.I. refers to plant introduction accession number.

Table 10.--Incidence of IB in alsike clover varieties

Plant age (weeks)	Common alsike		P.I. 184687[1]	
	Plants	IB	Plants	IB
	Number	Percent	Number	Percent[2]
21	19	0	14	14
24	6	0	9	22
34	26	15	27	44
Total or mean	51	8	50	32*

1/ Introduced from Germany.
2 * Significantly different from common alsike, $P < 0.05$.

mice appeared to be similar to aging. To determine the effect of ionizing radiations on incidence of IB, seeds and young red clover plants were subjected to varying dosages of gamma rays or thermal neutrons or both at Brookhaven National Laboratory, Upton, N. Y.

Pennscott seeds in 10-g. packets were subjected to gamma irradiation at dosages of 15,000, 20,000, 25,000, 30,000, and 35,000 r., and to thermal neutrons for 1, 3, 5, and 6 hours (table 11). Controls were included. The irradiated seeds were planted in potting soil 13 days later and maintained in the greenhouse at 17^{o} to 20^{o} C.

Generally, irradiation of seeds with gamma rays had little effect on the growth of seedlings or the incidence of IB. At the higher thermal neutron dosages, emerged seedlings apparently had no primary meristem and usually died within 2 weeks. At lower thermal neutron dosages and higher dosages of gamma rays, primary and trifoliolate leaves were initially mottled and distorted, but this effect was temporary. Analysis of variance of the data of table 11 did not reveal any significant effect of either gamma or thermal neutron irradiation of seeds on incidence of IB. There was a trend towards smaller crown size with increased thermal neutron dosage. However, at the two highest levels of thermal neutrons, the incidence of IB appeared to be increased in the few surviving plants.

Seven-week-old plants of the Pennscott variety and three IB-resistant selections were irradiated with thermal neutrons. Groups of unpotted plants, each containing 24 Pennscott and 10 plants of each IB-resistant selections, were irradiated for time periods ranging from 0.5 to 3 hours as shown in table 12. Roentgens absorbed by the plants were determined by foil readings. All plants survived the treatments.

Table 11.--Effect of irradiation of seeds with gamma rays and thermal neutrons on crown diameter, and incidence of IB in Pennscott red clover

Gamma rays	Plants[1/]	Mean crown diameter	Incidence of IB[2/]
Roentgens	Number	mm.	Percent
0 (control)	67	8.7	31
15,000	76	8.5	38
20,000	74	8.2	31
25,000	68	8.7	40
30,000	77	8.1	32
35,000	71	8.9	41
Total or mean	433	8.5	36
Thermal neutrons - duration (hr.):			
0 (control)	65	9.1	31
1	69	8.6	41
3	46	7.2	22
5	10	7.1	50
6	3	8.3	67
Total or mean	193	8.1	42

1/ Approximately equal numbers of plants were examined after 5 and 6 months of growth. Values are the total of two examinations.

2/ Differences not statistically significant, $P<0.05$.

Table 12.--Effect of irradiation of 7-week-old red clover plants with thermal neutrons on crown diameter, and incidence of IB, 28 weeks after treatment

Variety or strain	Duration of thermal neutron treatment (hr.) 0.0	0.5	1.0	1.5	2.0	2.5	3.0	Total or mean
Pennscott (unselected):								
Number of plants	21	24	24	23	24	21	24	161
$\overline{X}$ crown diam. mm.	9.3	9.3	9.5	9.2	9.3	8.0	8.6	9.0
IB, percent	29	29	25	35	46	48	75	41[2/]
Pennscott IB-resistant 1A:								
Number of plants	18	10	9	10	10	9	10	76
$\overline{X}$ crown diam. mm.	8.6	8.0	8.8	8.0	7.4	6.8	7.1	7.8
IB, percent	0	0	22	10	20	33	10	12
Pennscott IB-resistant 4A:								
Number of plants	18	10	10	10	10	10	9	77
$\overline{X}$ crown diam. mm.	7.8	7.6	8.9	7.7	8.5	7.2	7.3	7.8
IB, percent	11	10	10	20	20	10	22	14
Iran IB-resistant:								
Number of plants	20	9	10	10	8	9	8	74
$\overline{X}$ crown diam. mm.	10.4	10.3	9.3	8.8	8.6	9.4	8.2	9.3
IB, percent	20	11	10	30	0	11	13	15
Treatments:								
Total plants	77	53	53	53	52	49	51	388
$\overline{X}$ crown diam. mm.	9.0	8.9	9.1	8.4	8.4	7.8	7.8	8.5
IB[1/], percent	16	17	19	26	29	31	43	25

1/ The percent increase in IB with increased dosage was highly significant ($P < 0.01$).

2/ Pennscott plants had significantly more IB ($P < 0.01$) than the IB-resistant selections.

Irradiation of plants with thermal neutrons resulted in yellowing of leaves, irregularly shaped leaves, and dwarfing. The latter two phenomena increased linearly with dosage level, while yellowing did not. There was also a slight decrease in crown diameter with increase in dosage levels. Statistical analysis of the data of table 12 indicated that the increase in incidence of IB with thermal neutron dosage was linear and highly significant. Pennscott plants had significantly more IB than the IB-resistant selections. Differences in incidence of IB between IB-resistant selections were not significant, and there was no significant interaction between radiation treatments and selections.

Crown diameters tended to decrease slightly with increase in thermal neutron dosage, whether applied to seeds or to young plants. When applied to seeds, the effect was lethal after 5 or 6 hours exposure (table 11). In irradiated plants, the increase in percent of IB was accompanied by a slight decrease in crown diameter, suggesting the deleterious effect of thermal neutrons on plant growth. This is in contradiction to the positive correlation between IB and crown size observed previously. It would appear that irradiation of plants with thermal neutrons enhances development of IB. If irradiation of plants with thermal neutrons accelerates the aging process, and if IB development was increased by this treatment, then it could be inferred that IB was related to aging.

Effect of Age of Seed

Plants were grown in pots of soil from seed from the lots listed in table 13. The unknown strain was related to the Pennscott variety. As indicated in table 13, there were only small differences in percentage of IB among the lots, but there was a trend toward more IB in plants grown from younger seed.

Table 13.--Effect of age of seed on incidence of IB in red clover

Variety	Age of seed	Age of plants (Months) 6 Plants	6 IB	7 1/2 Plants	7 1/2 IB	Total plants	IB 1/
	Years	Number	Percent	Number	Percent	Number	Percent
Unknown	20	25	32	27	50	52	41
Pennscott	9	24	21	31	71	55	49
Pennscott	1	34	41	31	71	65	55
Total or mean		83	33	89	65	172	49

1/ Differences not statistically significant, P <0.05.

Incidence of IB in Relation to Crown Diameter and Age of Plants

Results of numerous greenhouse and field experiments indicated that most treatments had little direct effect on incidence of IB. But a trend toward greater incidence of IB was observed among plants in treatments more favorable to plant growth as indicated by crown diameter. To determine the relationship of both crown diameter and plant age to the incidence of IB, data from more than 20 greenhouse experiments were compiled and analyzed.

The number and percentage of plants with IB for each two-way classification are presented in table 14. Because the numbers of plants involved in each two-way classification were unequal, the method of unweighted means as described by Snedecor and Cochran (1967) was used for the analysis of variance. Results show conclusively that incidence of IB is closely associated with crown size, and that age had very little influence on IB in plants of the same crown diameter. This relationship is depicted graphically in figure 2.

Discussion and Conclusions

Evidence obtained from these studies indicates that IB in red clover is not incited by a pathogenic fungus or bacterium, but is a physiogenic disease associated with the rate of growth (size) of the taproot adjacent to the crown. This latter conclusion agrees with that of several other workers (Leffel and Graham, 1966; Willis, 1966; Cressman, 1967). In this research and in that of Cressman (1967), the incidence of IB was positively correlated with crown size, regardless of the chronological age of the plant.

Knowledge of this relationship should enable one to predict fairly accurately the percentage incidence of IB in plants of a given crown diameter. One is also led to conclude that vigorous, productive plants have a virtual built-in deterioration of the crown and taproot. Under field conditions, this factor in conjunction with external damage by root insects, diseases, and other adverse factors, aids in destroying the taproot, and thus limits the productive life of the plant. Survival then depends upon the capacity of the plant to form a secondary root system at the crown level, as indicated in previous reports (Newton and Graham, 1960; Graham, Newton, and Zeiders, 1965; Cressman, 1967).

Selection studies have shown that resistance or susceptibility to IB can be readily obtained. This is probably the most significant result of these experiments. At present, the value of selection for resistance as a means of increasing the persistence of red clover stands has not been demonstrated. Further study of the physiology, biochemistry, and genetics of IB-resistant and IB-susceptible selections seems to offer the best prospects for future progress. The relationships of vigor, yielding ability, and persistence of selections and their progeny should be investigated to see if agronomically desirable strains with reduced proneness to IB can be found.

Table 14.--Percentage incidence of IB in red clover in relation to crown diameter and age of plant. Data compiled from more than 20 greenhouse experiments

Crown diam. (mm.) (Mid-class)1/	Age in months when examined (Mid-class)2/ 4.5 Plants	4.5 IB	6.5 Plants	6.5 IB	8.5 Plants	8.5 IB	Total plants	IB
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
5.5	244	16	183	14	55	20	482	16
7.5	352	22	420	27	181	22	953	24
9.5	298	35	507	38	287	29	1,092	35
11.5	166	51	304	54	259	53	729	53
13.5	50	68	123	67	155	66	328	67
15.5	20	75	35	74	73	90	128	84
Total or mean	1,130	31	1,572	39	1,010	44	3,712	38

Analysis of Variance Summary Using the Method of Unweighted Means

Source of variation	df	Mean square	F-ratio
"Total"	17	0.0565	---
Crown size	5	.1873	89.19**
Age	2	.0007	1.0 N.S.
C X A	10	.0023	1.10 N.S.
Error	3,694	.0021	

1/ 2-mm. class intervals.
2/ 2-month class intervals.

** Significant at $P < 0.01$. N.S. = Nonsignificant.

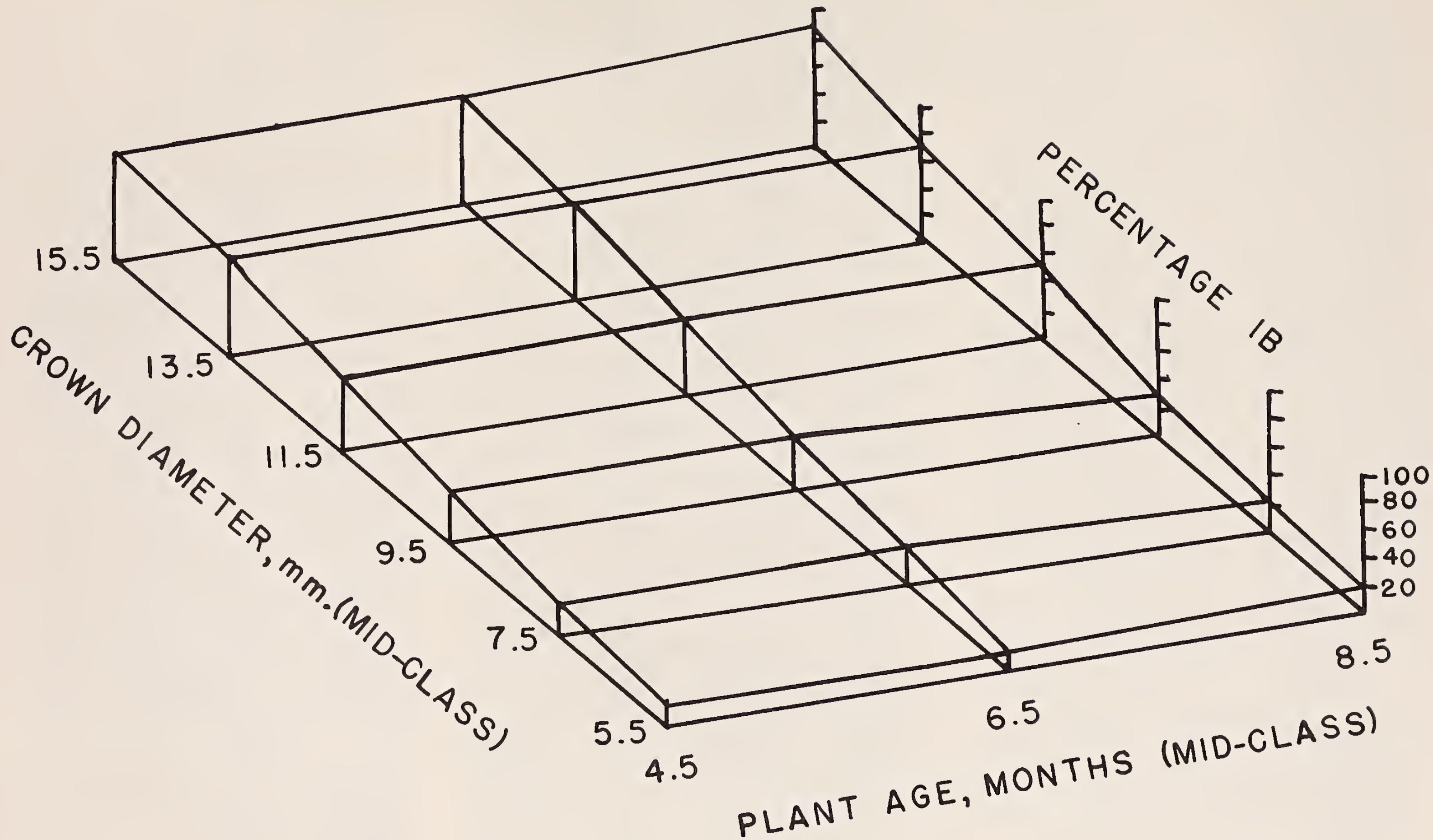

Figure 2.--Incidence of IB in red clover in relation to crown diameter and age of plants. From data of table 14. Total plants examined = 3,712.

Plants grown at 17^{o}C. had larger crowns and a higher percentage IB than plants grown at 27^{o}. The stress of frequent clipping caused highly significant reductions in crown size at both 17^{o} and 27^{o}. Conversely, plants grown under more favorable conditions of infrequent clipping had much larger crowns and significantly more IB at both temperatures. These results illustrate the relationship between favorable growing conditions, crown size, and incidence of IB.

Studies at this laboratory on the role of micro-organisms, which included isolations from IB and non-IB crown tissue, the inoculation of red clover plants with isolated micro-organisms, the growth of red clover plants under aseptic or gnotobiotic conditions, and the histology of IB tissues (unpublished), indicate that fungi and bacteria are not causal agents of IB. As a result of histological studies of IB in red clover, Cressman (1967) also ruled out fungi and bacteria as causal agents, but suggested that a virus might be involved.

The possibility of IB being caused by a virus was not investigated. Throughout the experiments, occasional plants with virus symptoms (usually red clover vein mosaic) were observed, but there was no indication that virus plants had more or less IB than nonvirus plants. Internal browning of tomato fruits, a disease characterized by necrotic breakdown of parenchyma tissues similar to IB of red clover, has been shown to be caused by a virus (Boyle and Wharton, 1957; Wharton and Boyle, 1957); soil moisture and cultivar susceptibility were also reported to affect incidence. From the similarities in symptoms, it might be inferred that a virus could be involved in development of IB in red clover. If a virus were involved, external symptoms have not as yet been detected to indicate its presence. The fact that IB has been found in red clover wherever it is grown suggests that a virus would have to be ubiquitous and without external symptoms. The occurrence of IB in red clover plants grown in aseptic isolators suggests that if a virus were involved, it would be seed-borne.

Summary

Extensive greenhouse experiments have been conducted to determine the cause of internal breakdown (IB) in crowns of red clover or factors affecting its development. The cause of IB was not associated with a pathogenic fungus or bacterium, mineral nutrient imbalance, the action of plant growth regulators, temperature, or day length. Incidence of IB was increased by irradiation of young plants with thermal neutrons. Both resistance and susceptibility to IB were obtained by selection and crossing. Most treatments had little direct effect on incidence of IB, but more IB occurred in those treatments that favored growth of the plants. Incidence of IB was positively correlated with crown diameter, regardless of the chronological age of the plant. Incidence of IB in red clover varieties was similar. IB also developed in crowns of alsike clover (*Trifolium hybridum* L.)

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